

A new pheretimoid earthworm of the genus *Metaphire* Sims & Easton, 1972 (Oligochaeta, Megascolecidae) from northeastern China with data from the mitochondrial genome

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Abstract

A new pheretimoid earthworm species, *Metaphire liaoningensis* **sp. nov.**, was investigated in northeastern China. The taxonomy of *M. liaoningensis* **sp. nov.** using morphological characters and molecular data is reported, as well as its current geographical range in northeastern China. The new species, belonging to the *M. houlleti* species group, is characterized by having three pairs of spermathecal pores in 6/7/8/9, round and small pre-clitellar genital markings medio-ventral to the spermathecal pore opening, the first dorsal pore in 12/13, and the spermathecae ampulla large, nearly elliptical with a surface wrinkled and a duct long and stout. The molecular phylogenetic position of *M. liaoningensis* **sp. nov.** within the *M. houlleti* species group is performed by COI and mitogenomic data, respectively. The COI K2P genetic distance between the new species and the other species within the *M. houlleti* species group was at least 16%. This is the second updated report of a new pheretimoid species in northeastern China since the last decade.

Key Words

Biodiversity, new species, Northeast China, taxonomy

Introduction

It is well known that China is one of the countries in Asia having rich species diversity of earthworm fauna. To date, an estimated total of 640 species and subspecies of earthworms from nine families and 28 genera have been recorded in China (Huang et al. 2006; Aspe 2016; Jiang 2016;

Shen et al. 2016, 2019; Zhao et al. 2017; Dong et al. 2018, 2019, 2024; Jiang and Qiu 2018; Jiang et al. 2018; Sun et al. 2018, 2021; Yuan et al. 2019; Han et al. 2024; Jin et al. 2024; Li et al. 2024); this number has nearly doubled in the last 16 years since Blakemore's recorded lists (Blakemore 2007a, 2008). The Megascolecidae family accounts for more than 90% of earthworm species in China, with an

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estimated total of more than 600 species and subspecies recorded, making it the most dominant earthworm family in the country. *Amyntas*, with around 476 species and subspecies, and *Metaphire*, with around 135 species and subspecies, are the most speciose genera in this family, accounting for 89% of the total number of species in China.

Megascolecidae species showed an uneven distribution in China. Recent statistical data revealed larger occurrences in southern China, with more than 79 species recorded in each of the provinces of Guangxi, Hainan, Sichuan, and Taiwan, while only 13 species were recorded in the whole northern region (most are considered widespread species with higher adaptability). Although this phenomenon may be due to more favorable temperature and humidity in the southern regions, it may also be related to the insufficient sampling efforts invested in taxonomic studies in the northern part of the country (Jiang 2016). Moreover, Dong et al. (2024) have recently identified three new earthworm species in eastern and southern China, which includes one *Metaphire* species (*M. changbaimontis* Dong & Shen, 2024) located in the Changbai Mountain in Northeast China. This marked the first discovery of a pheretimoid species since the previous decades (Yu et al. 1992).

A recent investigation conducted in 2023 in Northeast China has led to the discovery of a new species belonging to the pheretimoid earthworm group of the genus *Metaphire*. Live specimens of these species share similar external characteristics to those of *M. tschiliensis* (Michaelsen, 1928) and *M. guillelmi* (Michaelsen, 1895) in terms of body size and pigmentation (updated taxonomic studies of these two species are ongoing). However, at present, the major range of occurrence of this new pheretimoid earthworm species is limited in northeastern China only to the province of Liaoning and east of the Inner Mongolia Autonomous Region.

This work therefore aims to report the taxonomy of *M. liaoningensis* sp. nov. using morphological characters and molecular data. The report of this new species will provide additional information on the earthworm diversity of Northeast China as well as updated information on the pheretimoid earthworm fauna in the country.

Materials and methods

Earthworm specimens were collected in the summer of 2023, around the months of May and July, in Northeast China that includes the provinces of Heilongjiang, Jilin, and Liaoning and East of Inner Mongolia Autonomous Region. The collection sites chosen were mainly based on three habitat types, including forests, farmlands, and urban parks. Earthworms were collected through digging and hand sorting. Collections near the sites with surface castings were also taken into account. Earthworm specimens were then preserved in 100% ethanol in the field and stored at -20°C in the laboratory. Out of over 200 pheretimoid earthworm individuals collected in this study, a total of 11 individuals of *M. liaoningensis* were

morphologically and genetically identified (Table 1), while the rest of the pheretimoid earthworm species are reported separately in ongoing studies.

Earthworm external and internal morphological characters were examined using a stereomicroscope (ZEISS) and ZEN 3.3. pro software for image capturing and to aid in identifying and measuring small organs and other characters for morphological analysis. The generic diagnoses and taxonomic assignments follow Sims and Easton (1972) and Xiao (2019).

Total genomic DNA was extracted from the posterior part of the specimen using the TIANamp Genomic DNA Kit (China). Four mitogenomic data were obtained from *M. liaoningensis* sp. nov., *M. tschiliensis*, and *M. guillelmi* using high-throughput sequences with a paired-end 150 bp strategy on the platform of DNBseq in BGI Genomics (China). Clean data was filtered from the raw data following the steps of Zhao et al. (2022). The whole mitogenomic sequence was assembled by MitoZ v2.4 (Meng et al. 2019) and annotated by the online tool MITOS2 (https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu%2Frepos%2Fuc%2Fmitos2%2Fmitos2%2F2.1.3%20galaxy0) and checked manually by comparing the published mitogenomes of *Metaphire*. The four mitogenomic sequences and annotation information were submitted to GenBank (accession numbers see Table 1).

Mitogenomic phylogenetic analyses based on the two datasets of 13 protein-coding genes (PCGs), including the 3rd site of codon (named Site123) and excluding the 3rd site of codon (named Site12), were performed using Bayesian inference (BI) and maximum likelihood (ML) methods with a concatenated and partitioned strategies. The best DNA substitute model was chosen using jModelTest 2.1 (Darriba et al. 2012), and GTR + I + G was the best model based on the Akaike information criterion. BI was performed by MrBayes 3.2.7a (Ronquist et al. 2012); two million generations were run to make the average standard deviation of split frequencies less than 0.01. ML analysis was performed in RAxML 8.0 (Stamatakis 2014) using the default rapid hill-climbing algorithm and the GTRGAMMA model to search for the best tree, and the clade support was assessed using 1000 rapid bootstrap replicates. *Perionyx excavatus* was set as the outgroup in both BI and ML analyses.

The former region of the cytochrome c oxidase subunit I (COI) was amplified using the primers LCO1490 (5-GGTCAACAAATCATAAAGATATTGG-3) (Folmer et al. 1994) and COIE (5-TATACTTCTGGGTGTC-CGAAGAATCA-3) (Bely and Wray 2004) through polymerase chain reaction (PCR). The mixture (total volume 25 μl) contained 1 μl DNA and 17.25 μl sterile dH_2O , 2.0 μl of dNTP, 2.5 μl of buffer, 0.25 μl Easy Taq Polymerase (TransGen Biotech Co., LTD, Beijing, China), 1.0 μl of former primer LCO1490, and 1.0 μl of reverse primer COIE. The cycling profile was as follows: initial denaturation for 5 min at 95°C ; then denaturation for 30 sec at 95°C , annealing for 30 sec at 45°C , and extension for 30 sec at 72°C for 35 cycles; and final extension

Table 1. Specimens information provided here and available online DNA data analyzed in this study (AR is the abbreviation of autonomous region).

Specimen ID	Species	Location	GPS Coordinates (Latitude, Longitude)	Genetic Marker	Accession No.
368R6_01	<i>M. liaoningensis</i> sp. nov.	Liaoning Prov., Shenyang Pref., Wanquan Park	41.7884°N, 123.4659°E	COI	PP527726
368R6_02	<i>M. liaoningensis</i> sp. nov.	Liaoning Prov., Shenyang Pref., Wanquan Park	41.7884°N, 123.4659°E	COI	PP527727
368R6_05	<i>M. liaoningensis</i> sp. nov.	Liaoning Prov., Shenyang Pref., Wanquan Park	41.7884°N, 123.4659°E	COI	PP527730
368R7_01	<i>M. liaoningensis</i> sp. nov.	Liaoning Prov., Shenyang Pref., Wanquan Park	41.7884°N, 123.4659°E	COI	PP527731
368R7_03	<i>M. liaoningensis</i> sp. nov.	Liaoning Prov., Shenyang Pref., Wanquan Park	41.7884°N, 123.4659°E	COI	PP527733
359R4_03	<i>M. liaoningensis</i> sp. nov.	Liaoning Prov., Shenyang Pref., Zhongshan Park	41.7835°N, 123.4012°E	COI	PP527718
359R4_05	<i>M. liaoningensis</i> sp. nov.	Liaoning Prov., Shenyang Pref., Zhongshan Park	41.7835°N, 123.4012°E	COI	PP527719
359R5_03	<i>M. liaoningensis</i> sp. nov.	Liaoning Prov., Shenyang Pref., Zhongshan Park	41.7835°N, 123.4012°E	COI	PP527723
551_03	<i>M. liaoningensis</i> sp. nov.	Liaoning Prov., Huludao Pref., Longwan Park	40.7143°N, 120.8415°E	COI mitogenome	PP527737 PP504837
319R11_04	<i>M. liaoningensis</i> sp. nov.	Inner Mongolia AR, Tongliao Pref., People's Park	43.6065°N, 122.2663°E	COI	PP527712
319R31_04	<i>M. liaoningensis</i> sp. nov.	Inner Mongolia AR, Tongliao Pref., People's Park	43.6065°N, 122.2663°E	COI	PP527714
319R31_01	<i>M. liaoningensis</i> sp. nov.	Inner Mongolia AR, Tongliao Pref., People's Park	43.5964°N, 122.2847°E	mitogenome	PP507115
319R11_01	<i>M. tschiliensis</i>	Inner Mongolia AR, Tongliao Pref., People's Park	43.5964°N, 122.2847°E	COI	PP527386
374NH1_005	<i>M. tschiliensis</i>	Jilin Prov., Changchun Pref., Nanhu Park	43.8564°N, 125.3067°E	COI	PP527433
HNLN-GR12_10	<i>M. tschiliensis</i>	Henan Prov., Luoyang Pref., Luoning county, Tobacco field	34.4363°N, 111.6398°E	mitogenome	PP527650
LFSF_011	<i>M. guillelmi</i>	Hebei Prov., Langfang Pref., Langfang Normal University	39.5222°N, 116.6655°E	COI mitogenome	PP527738 PQ164794
-	<i>M. guillelmi</i>	-	-	COI	KT429017
-	<i>M. houlleti</i>	-	-	COI	KU565273
-	<i>M. houlleti</i>	-	-	COI	MF481209
-	<i>M. houlleti</i>	-	-	COI	OP787155
-	<i>M. sanmingensis</i>	-	-	COI	KY774384
-	<i>M. acampanulata</i>	-	-	COI	OP787172
-	<i>Polypheretima elongata</i>	-	-	COI	LC762551
-	<i>Perionyx excavatus</i>	-	-	COI	EF494507

for 5 min at 72 °C. Positive PCR products were sent to Tianyi Huiyuan Biotechnology Co., Ltd. (Beijing, China) for Sanger sequencing. Fasta sequences of COI were submitted to GenBank (accession numbers see Table 1). COI sequences of other pheretimoid species included in the analysis were downloaded from GenBank (accession numbers see Table 1). Genetic distances were calculated using the Kimura 2 parameter (K2P) model (Kimura 1980) performed in MEGA 5.0 (Tamura et al. 2011).

Results and discussion

Taxonomy

Family Megascolecidae Rosa, 1891
Genus *Metaphire* Sims & Easton, 1972

Metaphire liaoningensis Han & Zhao, sp. nov.

<https://zoobank.org/EB6E1CC0-73A9-4078-A3DD-F47A1E5D223A>
Figs 1, 2

Material examined. Holotype: • 1 clitellate (368R6_02), Wanquan Park (41.7884°N, 123.4659°E, 57 m elev.), Shenyang Prefecture, Liaoning Province, 2023-07-

01. **Paratypes:** • 4 clitellates (368R6_01, 368R6_04, 368R7_01, 368R7_02), same data as of holotype; • 1 clitellate (359R4_05), Zhongshan Park (41.7835°N, 123.4012°E, 60.4 m elev.), Shenyang Prefecture, Liaoning Province, 2023-07-01. **Other specimens:** • 2 juveniles (359R4_03, 359R5_03), the same with 359R4_05; • 1 juvenile (551_13), Shuangshu Town (40.7060°N, 120.7910°E, 25.0 m elev.), Huludao Prefecture, Liaoning Province, 2023-07-27; • 2 juveniles (319R11_04, 319R31_04), People's Park (43.5964°N, 122.2848°E, 183.5 m elev.), Tongliao Prefecture, Inner Mongolia Autonomous Region, 2023-07-24. All above specimens were collected by Yufeng Zhang, Shixiong Ma, Min Liu, and Mingyan Qin. All the specimens are stored at the Hebei Key Laboratory of Animal Diversity, Langfang Normal University, Langfang, China (C-HLU).

Etymology. The specific name refers to the first known distribution range, which is in the Liaoning Province, Northeast China.

Diagnosis. Medium sized, length 125–200 mm, diameter 6.0–7.0 mm, number of segments 120–145. Prostomium epilobic. First dorsal pore on 12/13. Three pairs of spermathecal pores in 6/7/8/9, ventrolaterally positioned. Male pore in XVIII in copulatory pouches (everted); ventral distance between openings of the pouches about 3.4–

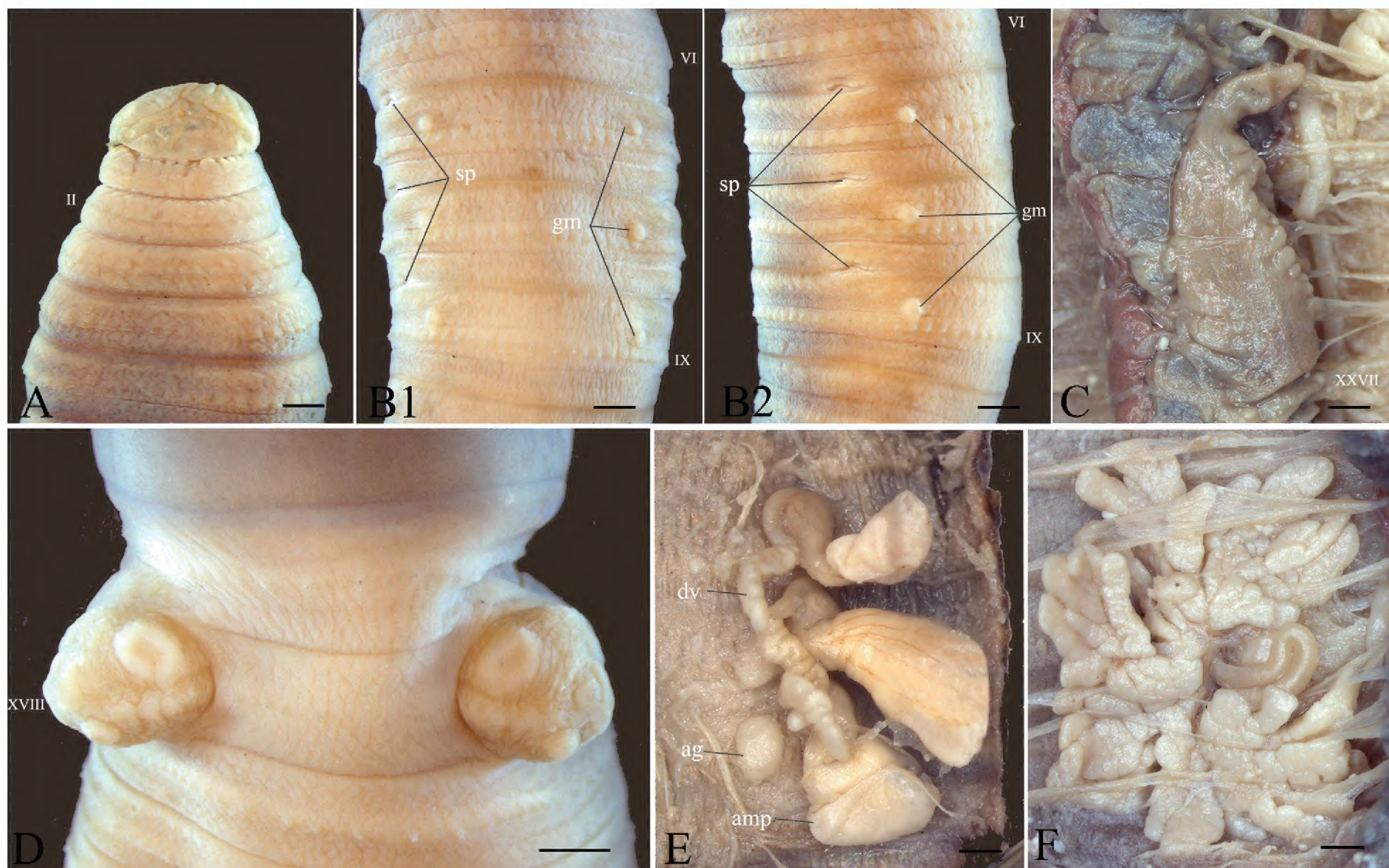


Figure 1. *Metaphire liaoningensis* sp. nov., holotype (368R6_02). **A.** Dorsal view of the prostomium; **B1.** Ventral view of the spermathecal region; **B2.** Lateral view of the left spermathecal region; **C.** Intestinal caeca; **D.** Copulatory pouches (everted); **E.** Right spermathecae; **F.** Right prostate gland. Scale bars: 1 mm.

4.5 mm. Preclitellar genital markings small and prominently rounded. Intestinal caeca simple. Septa 8/9/10 absent. Spermathecae ampulla nearly elliptical, large, surface wrinkled, ampulla duct long, stout with a swollen basal portion. Diverticulum originating from below the swollen portion of the spermathecal duct, stalk slender at the proximal end, enlarged and greatly coiled toward distal end. Accessory glands, sessile, small and round. Prostate glands paired in XVIII, very follicular, extending anteriorly to XV (or XVI) and posteriorly to XX (or XXI), divided into many finger-shaped pieces. Prostatic duct U-shaped and slender.

Description. External characters. Length 125–200 mm ($n = 6$). Color of preserved specimens may vary in shades because of the duration of preservation, but generally, dorsum region brown, covering half of the body length and fading to lighter brown towards posterior end with darker brown clitellum, while ventrum part is yellowish brown. Width 6.0–7.0 mm, segments 120–145. Prostomium epilobous (Fig. 1A). First dorsal pore on 12/13. Clitellum annular at XIV–XVI; setae or dorsal pores and intersegmental furrow absent. Setal arrangement perichaetine; setae number 43 (V), 56 (IX), 70 (XIII). Setae between male porophores: 12–20 (20 setae count if reaching on porophore). Female pore single in slit-like opening, medioventral at XIV.

Spermathecal pores three pairs, 6/7–8/9, ventrolateral; each pore is located inside a transverse slit cave-

like opening with ridges in the lip opening, 0.28–0.30 C apart ventrally. Pre-clitellar genital markings small and prominently rounded with diameters ranging from 0.3 to 0.6 mm, presetal on VII, XIII, and IX, 2–6 genital markings (rarely absent or with inconspicuous genital markings), paired or unilateral (rarely), located medio-ventral to spermathecal pore slit opening, widely spaced 0.21–0.26 C apart ventrally (Fig. 1B).

Male pores paired in XVIII (Fig. 1D), situated ventro-laterally at everted copulatory pouches (diameters ranging 1.8–2.5 mm); ventral distance between openings of the pouches 3.4–4.5 mm. All specimens displayed everted copulatory pouches and 2–3 male pore tubercles in each porophore (upper tubercles larger than lower tubercles). Post-clitellar genital marking absent.

Internal characters. Septa 8/9/10 aborted, 5/6/7/8 thick and muscular, 10/11/12/13 uniform thickness, 13/14 slightly thickened. Gizzard within IX–X (or only IX), small. Intestine enlarged from XV. Intestinal caeca are simple, originating in XXVII and extending anteriorly to XXIII (Fig. 1C); however, some caeca extend up until XX. Last pair of hearts in XIII.

Spermathecae (Fig. 1E) three pairs in VII–IX, ampulla nearly elliptical, large, surface flattened and wrinkled, 2.2–4.2 mm long, 2.0–2.8 mm wide; ampulla duct long, stout, 1.4–1.5 mm long, with a swollen basal portion, 1.2–1.3 mm wide; diverticulum originating from below the swollen portion of the spermathecal duct,

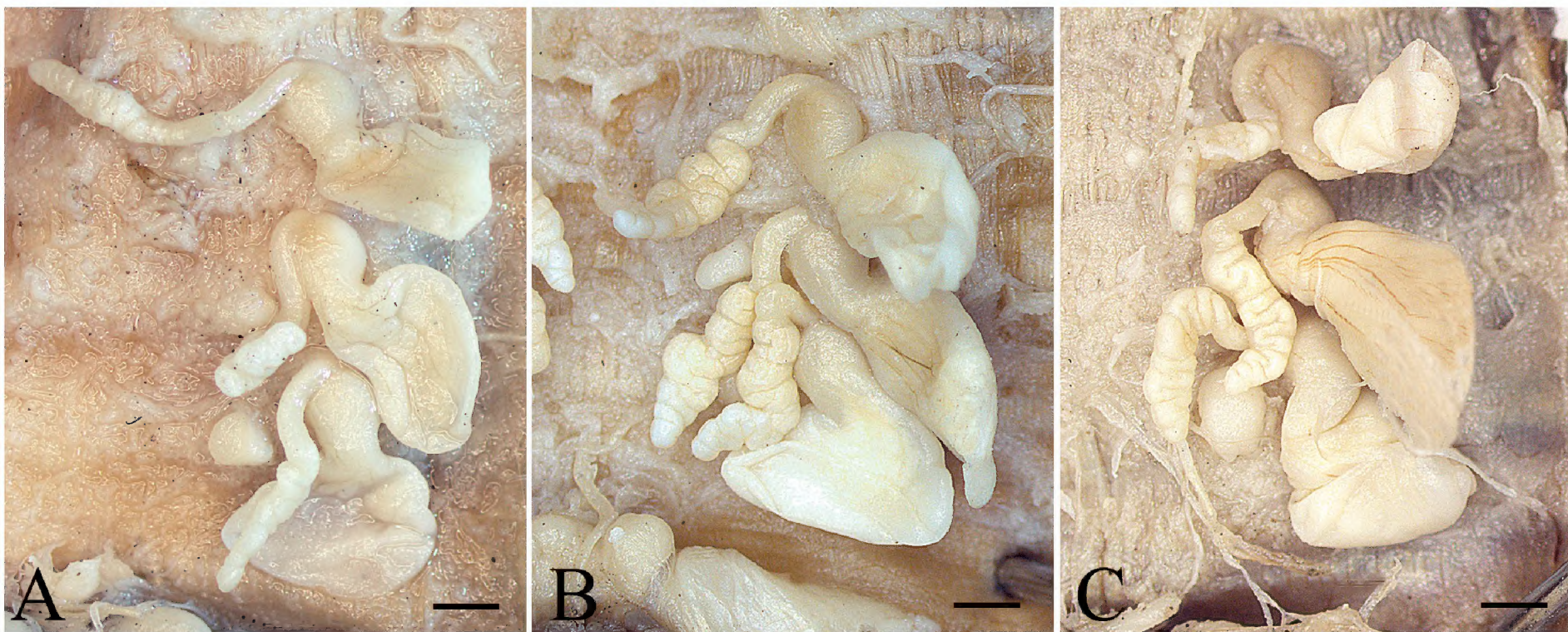


Figure 2. Variations in the spermatheca of *Metaphire liaoningensis* sp. nov. **A.** Poorly developed; **B.** Developed; **C.** Fully developed. Scale bars: 1 mm.

stalk slender at the proximal end 1.2–2.3 mm long, enlarged and greatly coiled toward distal end, receptacle is 2.2–3.6 mm long. Accessory glands, sessile, small and round. Variations of spermathecae are shown in Fig. 2.

Testis sac one pair, connected, in XI. Seminal vesicles are two pairs in XI and XII, well developed, follicular, posterior pair larger, each with a round or oval dorsal lobe. Prostate glands (Fig. 1F) large, paired in XVIII, follicular, divided into many finger-shaped pieces, extending anteriorly to XV (or XVI) and posteriorly to XX (or XXI). Prostatic duct U-shaped, slender.

Distribution. Northeastern China (Liaoning province and eastern Inner Mongolia Autonomous Region).

Habitats. Municipal parks.

Remarks. *M. liaoningensis* sp. nov. is classified into the *M. houlleti* species group (Sims and Easton 1972), which includes an estimate of 47 named species (Blakemore 2016). This group is characterized by having three pairs of spermathecal pores in 6/7/8/9, a male pore on XVIII, bithecate, and post-clitellar genital markings absent (Sims and Easton 1972). Currently, China has recognized 23 species belonging to the *M. houlleti* species group, of which 20 species were recorded by Xiao (2019) and *M. sanmingensis* by Sun et al. (2018), and two recently identified species, *M. donganensis* (Jin & Jiang, 2024) and *M. hanbaiduenensis* (Dong & Sun, 2024) from southern China. However, only 10 of these species have been recognized in Blakemore (2007b), mainly due to the known species recorded at that time and some changes at the genus and subspecies levels.

Collected specimens of the new species were all observed to have everted copulatory pouches and exhibit male pore tubercles. Table 2 presents a comparison of morphologically similar members of the *M. houlleti* species group in China with *M. liaoningensis* sp. nov. Among all the *M. houlleti* species group species in Chi-

na, the new species is clearly seen to be unique from the rest by having round and prominent pre-clitellar genital markings reaching up to a maximum of three pairs and having a large and flattened ampulla with a swollen base of the ampulla duct. Although live specimens of *M. liaoningensis* sp. nov. share similar outward morphological characters (such as size and pigmentation) with *M. tschiliensis*, internal structures differ, such as in the shape of the spermathecae ampulla, diverticulum length, and prostate gland structures.

In addition, *M. liaoningensis* sp. nov. differs from the other morphologically similar species with dorsal pore in 12/13 by the following specific traits: pre-clitellar genital markings present [vs. present or absent in *M. vulgaris agricola* (Chen, 1930) and *M. tschiliensis*], accessory glands present and sessile [vs. present and stalked in *M. kokoana* (Chen & Fong, 1975) and *M. aggera* (Kobayashi, 1934); vs. absent in *M. tschiliensis*, *M. guillelmi*; and vs. bundle of strands in *M. vulgaris agricola*].

Moreover, it may seem that the new species also has some internal similarities to *M. houlleti* (Perrier, 1872), such as the very follicular prostate gland with a slender U-shaped duct, yet the follicle coverage according to segment count and number differs. These differences may be attributed to the body size of the species, since *M. houlleti* specimens are smaller than those of the *M. liaoningensis* sp. nov. specimens.

Likewise, other species of the *M. houlleti* species group, which are non-endemic to China, such as *M. acampanulata* in Vietnam, which share similar size with *M. liaoningensis* sp. nov., also manifested obvious dissimilarities in both external and internal morphologies, such as the presence and count of genital markings on both pre-clitellar and post-clitellar regions, the presence of penial setae, the orientation of accessory glands, the ampulla shape and size, and the diverticulum length.

Table 2. Comparison of species belonging to the *M. houlleti* species group. The species are listed in order of size from largest to smallest.

Descriptions	<i>M. vulgaris agricola</i> (Chen, 1930)	<i>M. tschiliensis</i> (Michaelsen, 1928)	<i>M. vulgaris vulgaris</i> (Chen, 1930)	<i>M. acampanulata</i> (Nguyen)	<i>M. aggera</i> (Kobayashi, 1934)	<i>M. liaoningensis</i> sp. nov.	<i>M. kokoana</i> (Chen & Fong, 1975)	<i>M. guillelmi</i> (Michaelsen, 1895)
Length (mm)	154–240	115–240	120–215	98–198	175–198	125–190	107–160	96–150
Width (mm)	6–9	6.5–7	5–8	4.03–6.91	5.5–10	7	5.5–8	5–8
No. of segments	80–122	111–165	90–124	56–144	150–171	120–145	66–120	88–156
First dorsal pore	12/13	11/12 or 12/13	11/12	12/13	12/13	12/13	12/13	12/13 or 13/14
Setae on V, XIII, XX	-	45–50 (V), 70–72 (XIII)	-	-	-	43 (V), 70 (XIII)		44–64 (XX)
Sper. pore distance apart	-	0.29–0.30 C	-	0.36–0.50 C	-	0.28–0.30 C	½ circumference apart ventrally	-
Male pore distance apart	-	2/5 circumference apart	-	0.21–0.37 C	-	-	1/3 circumference apart ventrally	-
Setae between mp	16–20	10–17	12–22	8–25	16–23	12–20	13–20	14–21
Pre-clitellar gm	present or absent	present or absent	present	present	present	present	present	absent
Post-clitellar gm	absent	absent	present	present	present	absent	present	absent
Gizzard	IX–X	IX–X	IX–X	between 7/8 and 10/11	IX–X	IX–X	7/8	VIII and IX
Caeca	XXVII–XXIII	XXVII–XXIII	XXVII–XXIII	XXVII–XXIV	XXVII–XXIII	XXVII–XXIII (or some XX)	XXVII–XXII or XXIII	XXVII to XXII
Accessory glands	present	absent	present	present	present	present	present	absent

Table 3. Percentage of K2P distances of COI of *M. liaoningensis* sp. nov. and other members of the *M. houlleti* species group.

Species	1	2	3	4	5	6
1 <i>M. liaoningensis</i> sp. nov.	0–1%					
2 <i>M. tschiliensis</i>	17–19%	0–2%				
3 <i>M. guillelmi</i>	16%	19%	0%			
4 <i>M. houlleti</i>	20–22%	20–21%	21–24%	21–22%		
5 <i>M. sanmingensis</i>	22–23%	20%	20%	18–22%	0	
6 <i>M. acampanulata</i>	22%	17–18%	19%	10–18%	17%	0

Genetic analysis

Results of the K2P analysis using COI show that the interspecific distance between *M. liaoningensis* sp. nov. and species belonging to the *M. houlleti* species group ranges from 16% (*M. guillelmi*) to 23% (*M. sanmingensis*) (Table 3). Species divergence range of *M. liaoningensis* sp. nov. among other *M. houlleti* species groups revealed that values are higher than 13% as reported by Nguyen et al. (2021), which is considered to be the least genetic distance by Jeratthitikul et al. (2017). Interspecific K2P distances among *Metaphire* species reported in Vietnam are from 14.2% to 23.3%, with a mean distance of 17.7% (Nguyen et al. 2020). It is evident in the molecular characterization of the new species that it is distinct from the other members of the *M. houlleti* species group.

The four new sequenced mitogenomes here were visualized as Fig. 3. For the phylogenetic position of *M. liaoningensis* sp. nov. in the *M. guillelmi* species group,

two mitogenomic datasets showed different topologic relationships (Fig. 4). The Site123 dataset showed (*M. liaoningensis* sp. nov., (*M. guillelmi*, *M. tschiliensis*)), with a weak support of posterior probability (PP) of BI lower than 80 and bootstrap support (BS) value of ML lower than 60 (Fig. 4A). The Site12 dataset showed a topologic relationship of (*M. guillelmi*, (*M. liaoningensis* sp. nov., *M. tschiliensis*)), with a relatively high support value of PP = 94 and BS = 61 in both concatenated and partitioned strategies (Fig. 4B). For node x, the Site12 with exclusion of the third site of codon seems to improve the support value (Fig. 4) in comparison to Site123, and the possible reason is that a rapid substitute rate in the 3rd site of codon. However, the support value of Site12 still does not achieve a high support (e.g., PP > 95, BS > 70), as the phylogenetic position of *M. liaoningensis* sp. nov. is unsolved. By adding nuclear genes or more members of the *M. houlleti* species group in the future, it may be possible to resolve the phylogenetic position of the new species.

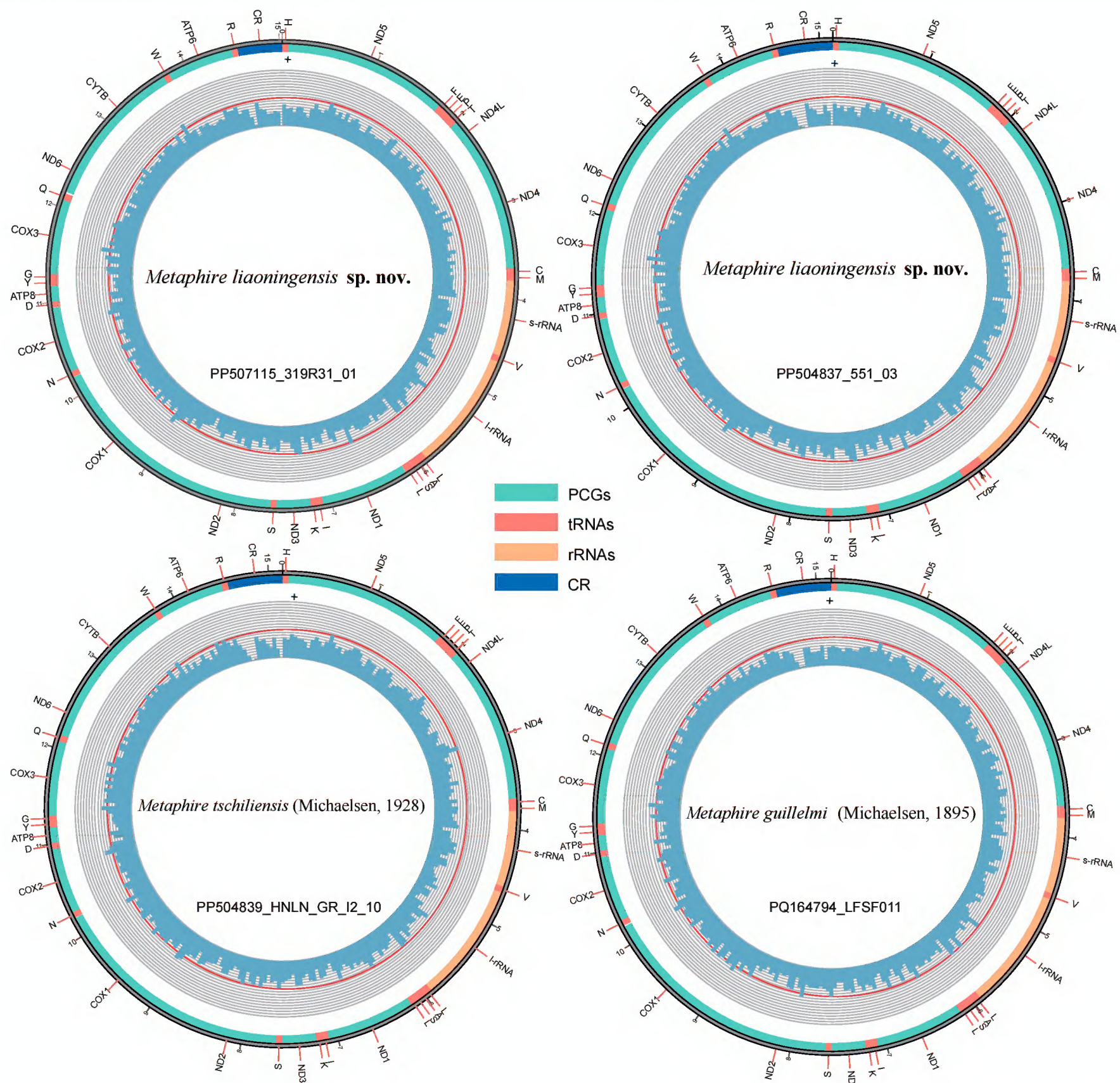


Figure 3. Map of mitogenomes of *M. liaoningensis* sp. nov., *M. guillelmi*, and *M. tschiliensis*. The inner circles indicate the GC content in every 50-site window, and the outer circle shows the arrangement of the genes; trnH was set as the start of the mitogenome. All genes are coded on the majority strand.

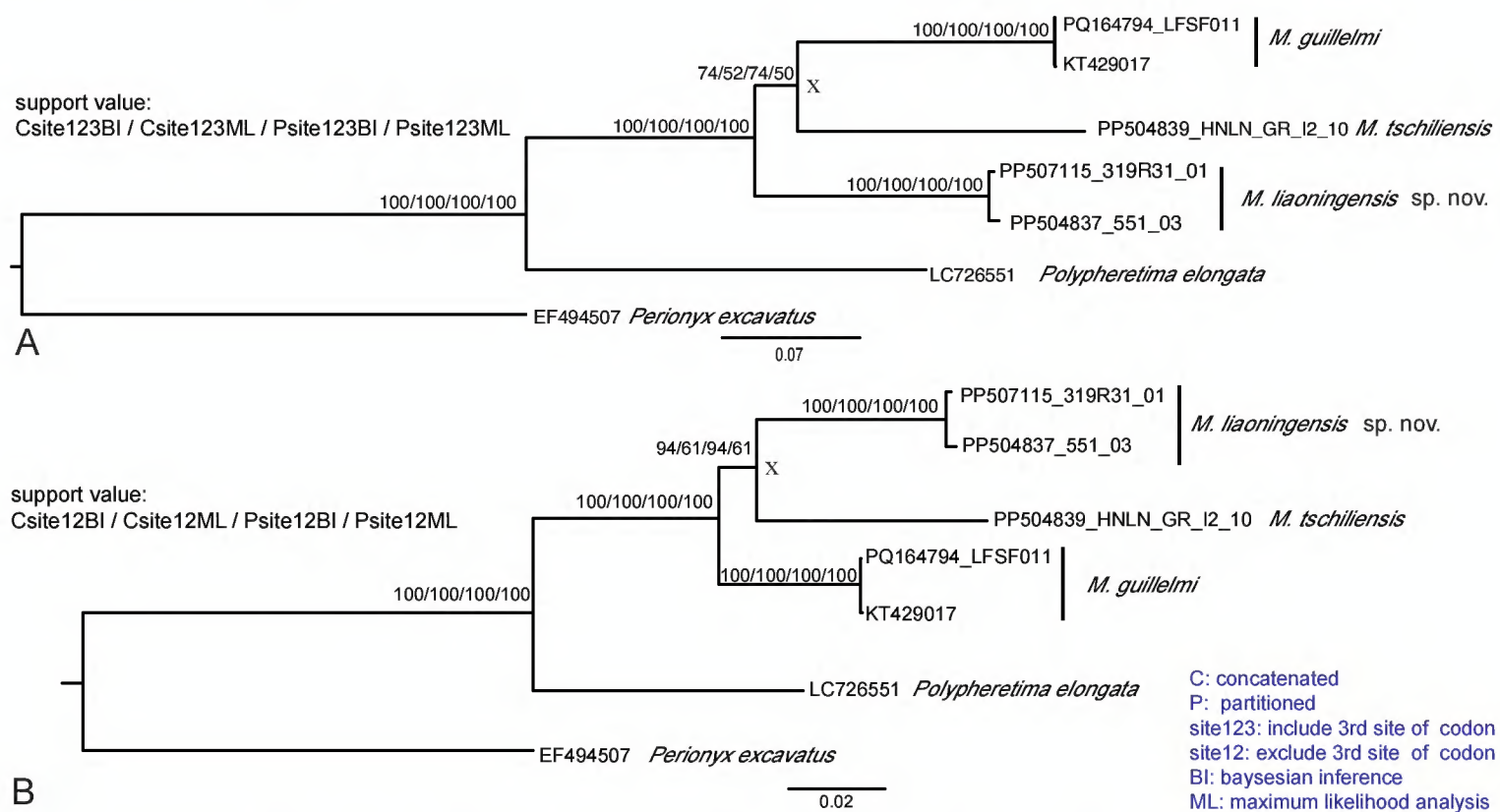


Figure 4. Phylogenetic relationship of *M. liaoningensis* sp. nov. and other species in the *M. houlleti* species group and outgroup taxa using the 13 coding genes of the mitogenome. **A.** The third site of codon is included; **B.** The third site of codon is excluded.

Conclusion

The discovery of *M. liaoningensis* sp. nov. in Northeast China is the second report of a new pheretimoid species in the region in the past decades. There has been an issue regarding an uneven distribution of pheretimoid earthworms in China, with less record in the northern part compared to the south. However, through the recent investigation and earthworm survey conducted last year, the discovery of *M. liaoningensis* sp. nov. and other pheretimoid earthworms in the region (on-going work) will lead to substantial information on recent earthworm fauna diversity in Northeast China and in the country as well. Still, more fieldwork in the future will be performed in northern China to further investigate the geographical range of *M. liaoningensis* sp. nov. and to broaden the pheretimoid earthworm survey in the region in aiming to discover newer endemic pheretimoid species in northern China.

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